Regulation of MicroRNA-221/222 Expression in Tamoxifen Resistant Breast Cancer

Senior Honors Thesis

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Problem

Tamoxifen is an effective and widely used drug in cancer therapy for women with breast cancer. The issue facing women who are treated with tamoxifen is that about 30% of them will have inherent or acquired resistance to this drug. We have shown that microRNA-221 and microRNA-222 are upregulated in tamoxifen resistant breast cancer and their overexpression has been shown to induce resistance to tamoxifen in a breast cancer cell line. The goal of this project is to elucidate the biochemical basis of deregulation of these miRNAs to better understand the mechanism of tamoxifen resistance, potentially revealing opportunity for therapeutic intervention.

Background

Breast cancer and tamoxifen

Breast cancer is the most common malignancy in women, accounting for 31% of all female cancers. Approximately 192,370 women were diagnosed with breast cancer in 2009 in the United States, and 40,170 will die of the disease [27]. Over two-thirds of breast cancers exhibit high expression of estrogen receptor, which contributes to tumor growth and progression. Blocking the steroid hormone pathway with the anti-estrogenic compound tamoxifen (TAM) and/or oophorectomy (the surgical removal of one or both ovaries) has been shown to be effective in this patient population and has been a widely used anti-cancer agent for over two decades. Tamoxifen acts as an estrogen antagonist in breast cancer and decreases the effects of estrogen by competitively binding to estrogen receptors. The Early Breast Cancer Trialists' Collaborative Group overview demonstrated a significant improvement in 15-year survival with

the addition of adjuvant TAM for 5 years following surgery [1]. Furthermore, TAM can also reduce the incidence of contra-lateral breast cancer and has been approved as a prophylactic agent to prevent breast cancer. Despite this accomplishment in the management of women with potentially endocrine-responsive breast cancers, about 30% of these women will experience disease progression due to either an intrinsic or acquired resistance to tamoxifen.

MicroRNAs

MicroRNAs (miRNA) are a recently discovered class of genes that are small, 18-25 nucleotide, non-coding RNA molecules which regulate expression of their target proteins. MiRNAs function by binding to the 3' UTR of their target mRNA, inhibiting translation and in some cases inducing rapid decay of the message [2].

The biogenesis of miRNAs begins with a primary transcript, which is transcribed from genomic DNA predominantly by RNA polymerase II. The active species is contained in a stem–loop structure that is excised by the nuclear ribonuclease III, Drosha. This stem–loop, termed the precursor, is exported from the nucleus. In the cytoplasm, the precursor is further processed by the RNase III enzyme Dicer to generate the mature miRNA species. The mature product is loaded into the RNA induced silencing complex (RISC), where it mediates translational suppression of the target mRNA (Figure 1)[3].

Figure 1

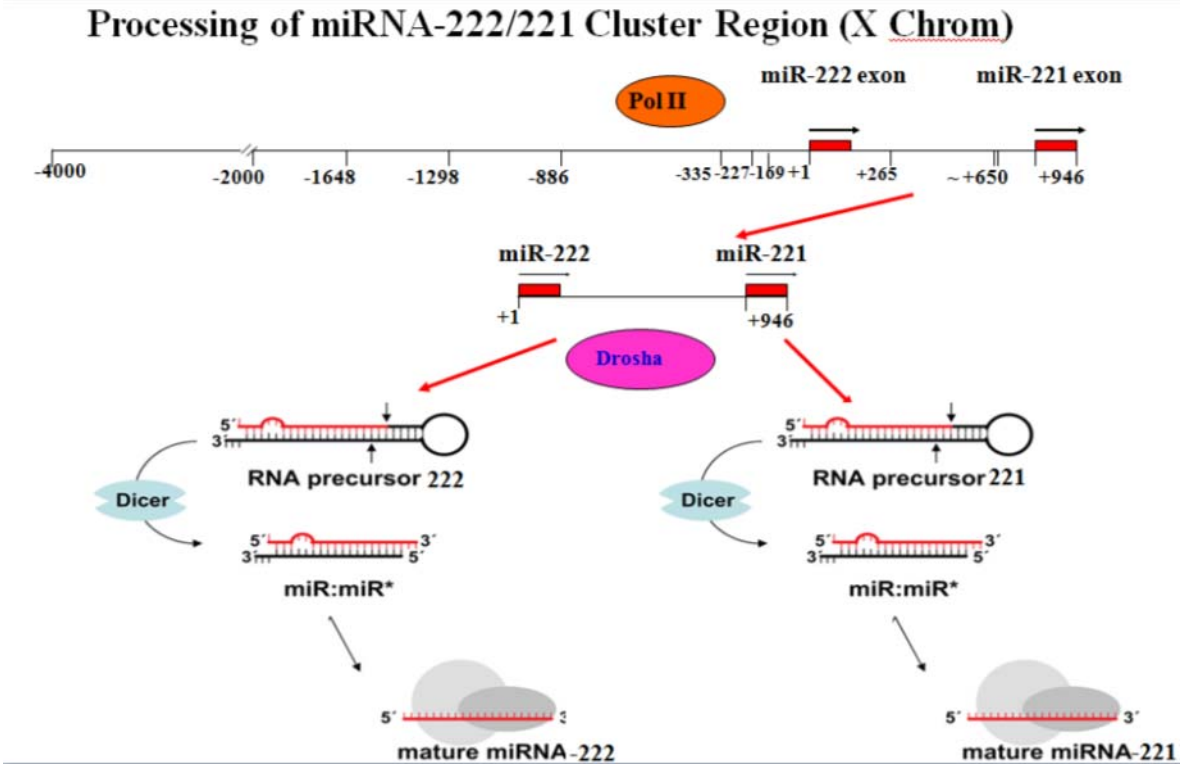


Figure 1 – Representation of the processing of the miRNA-221/222 cluster from primary transcript to precursor miRNA to mature miRNA loaded into RISC complex.

MicroRNAs in cancer and drug resistance

Each miRNA is thought to target multiple genes and recent studies have highlighted the key regulatory roles of miRNAs in all fundamental cellular processes in animals and plants, including carcinogenesis. Altered expression of miRNAs in primary human cancers including breast cancer has been used for tumor diagnosis, classification, staging, and prognosis [4]. A recent study with 76 neo-plastic and 34 normal breast tissue samples revealed altered expression of several miRNAs that could correctly predict the nature of the tumor analyzed [5].

Recent studies have also shown the critical role of miRNAs in conferring drug resistance or responsiveness in cancer. We have shown that increased expression of miRNA-221 and miRNA-222 increases resistance of MCF-7 cells to TAM [6], which was later independently validated by another group [7]. Increased levels of miRNA-21 in breast cancer [8] and

cholangiocarcinoma [9] have been attributed to increased resistance of these two tumor types to the chemotherapies, topotecan and gemcitabine, respectively. Similarly, increased miRNA-214 expression has been shown to confer cisplatin resistance in ovarian cancer [10].

MicroRNA regulation

Regulation of miRNA expression is a new and exciting field that continues to grow. Most miRNA studies thus far have explored the regulation of target genes by miRNAs or have studied the miRNA expression profiles of specific diseases, but very little information is available about regulation of miRNA expression. Currently three different mechanisms of miRNA regulation have been reported: epigenetic regulation through promoter silencing by methylation [11], transcriptional regulation through a variety of transcription factors [12,13] and regulation in the post-transcriptional processing of the miRNA primary transcript and precursor [3].

miRNA-221/222

MiRNA-221 and miRNA-222 are in a cluster on the X chromosome and are transcribed as a single primary transcript. They are then processed into two separate and distinct mature miRNAs, each with its own targets, many of which are identical. Although mature miRNA-221 and miRNA-222 have difference in their sequence, by virtue of having same seed sequence, they target almost identical proteins. They have been shown to be dysregulated in many types of cancer and are important in tumorigenesis [14, 15, 16]. In TAM resistant breast cancer we have shown that miRNA-221/222 play a critical role in the development of resistance by targeting p27/kip1, a cell cycle inhibitor [6]. When miRNA-221/222 downregulate p27/kip1, p27/kip1 can no longer bind to the CDK2/Cyclin-E complex, allowing the cells to progress through cell cycle and facilitating growth of cancerous cells, even when estrogen receptors are blocked by TAM (Figure 2)[6].

Figure 2

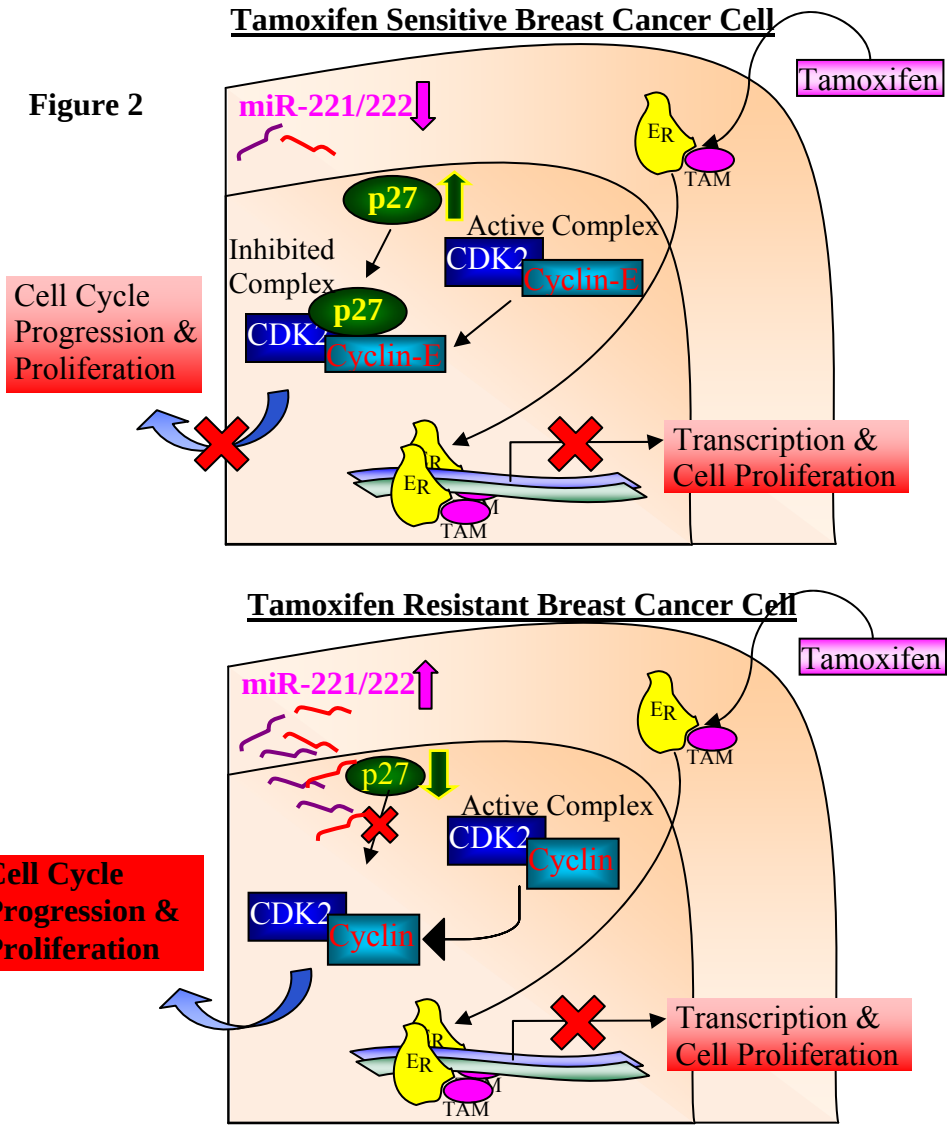


Figure 2 - Top MiRNA-221 and miRNA-222 expression is low in TAM sensitive cells, which allows p27 to effectively regulate the cell cycle. *Bottom* MiRNA-221 and 222 expression is high in TAM resistant cells, which decreases expression of their target molecule, p27. Without p27, the cells can continue to proliferate uncontrollably. In both diagrams, TAM is blocking estrogen–mediated cell proliferation.

The only reported regulation of these two miRNAs occurs in melanoma at the transcriptional level by the promyelocytic leukemia zinc finger protein, a known transcription factor [13]. However, there have been no reports of miRNA-221/222 regulation in tamoxifen resistant breast cancer. In earlier study, we showed that long-term treatment of breast cancer

cells with low concentrations of tamoxifen can increase miRNA-221/222 expression, which suggested a potential role of these two miRNAs in tamoxifen resistance. An unconfirmed report revealed that the transcription start site (TSS) for miRNA-221/222 was 15 kb upstream of the genomic region coding for miRNA-221/222 precursor [17]. Interestingly, using computer promoter analysis, we found that there was a CpG island within this upstream region. A CpG island is a region in the genome that contains a high frequency of CpG sites. A CpG site is the two base pair sequence CG with the "p" referring to phosphodiester bond between guanine and cytosine. In a CpG base pair the C-residue is susceptible to methylation, and when multiple CpGs in a CpG island are methylated, transcriptional silencing can occur [12]. CpG islands are in or near approximately 40% of mammalian genes and have a major role in regulating gene expression [29].

Another more recent report in March, 2010 revealed that the TSS was only about 230bp upstream of the genomic region for the miRNA-221/222 cluster [18]. The report also confirmed that the two microRNAs were transcribed together in a single 2.1kb primary transcript [18]. As transcriptional regulation can occur well upstream of the TSS, the CpG island would still have the potential to regulate miRNA-221/222 expression even if this newly reported TSS is correct.

Significance

Because about 30% of women who use tamoxifen world-wide are resistant to its anti-tumor effects, there is considerable interest in elucidating the molecular mechanisms of acquiring resistance to this important anti-cancer drug. Although altered expression of miRNAs in primary human cancers has been used for tumor diagnosis and prognosis, the potential involvement of miRNAs in induction of drug resistance, particularly tamoxifen resistance, has only recently

been explored. We demonstrated that miRNA-221/222 can induce TAM resistance in a breast cancer cell line by targeting the cell cycle inhibitor p27/kip1 [6]. This project will attempt to explore the regulatory mechanism that increases miRNA-221/222 expression, which may be the initial step in TAM resistance. Elucidation of the regulatory molecule(s) involved in miRNA-221/222 induced drug resistance is likely to unravel novel therapeutic targets and agents that restore responsiveness to TAM.

Working Hypothesis:

Based on differences in the level of miRNA-221 and miRNA-222 in tamoxifen resistant and sensitive cells, it can be speculated that miRNA-221 and miRNA-222 expression is differentially regulated in tamoxifen resistant cells versus tamoxifen sensitive cells. Transcriptional regulation, processing regulation and regulation by promoter methylation are currently the only three mechanisms of miRNA regulation reported and we believe miRNA-221/222 are regulated by one or more of these mechanisms. We will be able to examine all three types of regulation in our system to determine which type(s) is involved in the upregulation of miRNA-221/222.

Objectives:

Specific Aim 1: Identify candidate transcription factors involved in the upregulation of miRNA-221/222 in tamoxifen resistant cells.

Develop a model system where we can induce miRNA-221 and miRNA-222 expression using a MCF-7 breast cancer cell line to study transcriptional regulation and use this system to identify the promoter region that is involved in the differential regulation of miRNA-221/222

using luciferase reporter assays. Using these results, narrow down candidate transcription factors in the region of interest and test their regulatory ability in our system.

Specific Aim 2: Determine if there is a relationship between differential post-transcriptional processing and the upregulation of miRNA-221/222 in TAM resistant cells.

Detect the miRNA primary transcript and miRNA precursor using Northern blot analysis or PCR-based detection and use this data to determine if post-transcriptional regulation of miRNA-221/222 occurs in TAM resistant cells. This may occur during the processing of the miRNA-221/222 primary transcript or in the processing of miRNA-221 and miRNA-222 precursors.

Specific Aim 3: Investigate whether differential DNA methylation in the proximal or distal promoter is responsible for upregulating miRNA-221/222 expression.

Identify the methylation status of the CpG island in the regulatory region of both TAM sensitive and TAM resistant breast cancer cells. If TAM resistant cells exhibit a differential methylation pattern compared to TAM sensitive cells determine if demethylation of the CpG island can increase miRNA-221/222 expression.

Methodology and Rationale

Sp. Aim 1 - Identify candidate transcription factors involved in the upregulation of miRNA-221/222 in tamoxifen resistant cells.

Develop a model system to study transcriptional regulation

In order to study regulation of miRNA-221/222, we must first have a system where we can induce miRNA-221/222 expression. We can then use this system to study the cause of this induction. In our previous studies we used a TAM sensitive MCF-7 breast cancer cell line and a

TAM resistant MCF-7 cell line that were derived from a single TAM sensitive MCF-7 colony. The resistant cell line was created by treating the TAM sensitive cells with a low concentration of TAM for 6 months. We found that miRNA-221 and miRNA-222 expression was greatly increased in the resistant cells. Because both cell lines were derived from a single MCF-7 colony, any alteration in the miRNA profile should be the effect of prolonged TAM treatment. Therefore, we hypothesized that treating MCF-7 cells with TAM would induce miRNA-221/222 expression.

We treated TAM sensitive MCF-7 breast cancer cells with different concentrations of TAM for different lengths of time by adding TAM directly to the media. In one study, we treated the cells with high concentrations of TAM for periods of time ranging from 8 to 48 hours. In another study, we treated cells at low concentrations for different length of time, up to six months and harvested the cells at desired time points to isolate total RNA. We analyzed miRNA-221 and miRNA-222 expression levels using Real-Time RT-PCR to determine the change in response to TAM.

Identify promoter region involved in transcriptional regulation of miRNA-221/222

Transcription factors bind to the promoter region of a gene and act as transcriptional activators or repressors to regulate its transcription. Therefore, we must analyze the promoter region of miRNA-221/222 in order to study the transcriptional regulation of these genes. We must first identify the area of the promoter whose activity changes in response to TAM in order to identify the transcription factors involved.

We used luciferase reporter assays to analyze the miRNA-221/222 promoter. First, miRNA-221/22 promoter/luciferase reporter plasmids were created by cloning different lengths of the miRNA-221/222 promoter region directly in front of the luciferase coding sequence in

pGL3-Basic plasmid (Promega Corp). These plasmids were then transfected into TAM sensitive MCF-7 cells or OHTR cells. These cells were either treated with TAM or left untreated before analyzing their whole cell extract for luciferase activity with a luminometer. Because the luciferase gene is under the transcriptional control of the miRNA-221/222 promoter fragments, any increase in luciferase expression should be the result of an increase in the activity of that promoter fragment.

Sp. Aim 2 - Determine if there is a relationship between differential post-transcriptional processing and the upregulation of miRNA-221/222 in TAM resistant cells.

We believe there may be multiple levels of regulation for miRNA-221/222 expression, as there are multiple levels of regulation in many pathways, and that regulation may occur at the miRNA post-transcriptional level during its processing. We should be able to see if post-transcriptional regulation occurs using Northern Blotting techniques or PCR-based detection.

We first attempted to optimize a Northern blot technique to allow us to detect the primary transcript and precursors of miRNA-221/222. To do this, we isolated total RNA from TAM resistant MCF-7 cells that express high levels of miRNA-221/222. The RNA was size fractionated by running on polyacrylamide gel using gel electrophoresis and transferring the RNA to a Nylon membrane. We then probed this membrane with ^{32}P-labeled probes specific for a sequence in the miRNA-221/222 transcripts. The labeled membrane was exposed to a film and expression of the primary transcript and precursors should be visible. After we optimized the technique, we treated cells with and without TAM and perform the Northern blot analysis using the total RNA from these cells. We then compared the level of expression of the primary transcript and the precursors of miRNA-221/222 in TAM treated cells and untreated cells using the mature miRNA as a control to rule out transcriptional differences. For example, if the mature

miRNA is 12-fold higher in TAM treated cells than in untreated cells, and the primary transcript and precursors are also 12-fold higher in TAM treated cells, then the only difference in the miRNA-221/222 levels is due to an increase in transcription.

Another option to detect the miRNA primary transcript and precursor is to use a PCR-based detection system. Several primer pairs were designed to amplify the primary transcript. The reverse primer was used in place of a mixture of random primers during cDNA synthesis in order to enrich the cDNA pool for the primary transcript. The forward primer was then used with the reverse primer to amplify the primary transcript during RT PCR. This option has potential to be more sensitive, but the northern blot is the standard method used routinely.

Sp. Aim 3 - Investigate whether differential DNA methylation in the proximal or distal promoter is responsible for upregulating miRNA-221/222 expression.

Identify if there is differential DNA methylation

The first step in understanding if epigenetics is playing a role in the regulation of miRNA-221/222 expression is to identify the methylation status of the CpG island in the regulatory region in both TAM sensitive and TAM resistant breast cancer cells. If the miRNA-221/222 CpG island or the upstream regulatory region are more demethylated in the TAM resistant cells, which have a higher expression of miRNA-221/222, than TAM sensitive cells then this correlation indicates that methylation could be regulating miRNA-221/222 expression.

In order to identify the methylation status of the CpG island, we used combined bisulfate restriction analysis (COBRA). The principle behind COBRA is to treat genomic DNA from our cell of interest with bisulfite in order to distinguish between methylated and unmethylated CpGs. Bisulfite treatment converts all cytosine to thymine but does not affect 5-methylcytosine (the methylated C in a CpG). Thus, single-nucleotide resolution information about the methylation

status of CpGs can be determined with further testing. Once the DNA is converted, the region of the DNA that contains the CpG island is PCR amplified. This PCR product, which retains the bisulfite conversion information, is subjected to restriction enzyme digestion. The enzyme is selected so that the sequence of its recognition site is affected by the conversion of C to T. For example, the restriction enzyme Taq1 has a recognition site of TCGA. If the CpG in the sequence is methylation then the C will be retained and it will cut. If the CpG is not methylated, then the C will be converted to T, giving TTGA, and the enzyme will not recognize the sequence or cut at this site. Therefore, by using a selective restriction enzyme, and then running the digested product using gel electrophoresis to determine the difference in size, we could determine if a specific CpG is methylated (Figure 3).

Figure 3

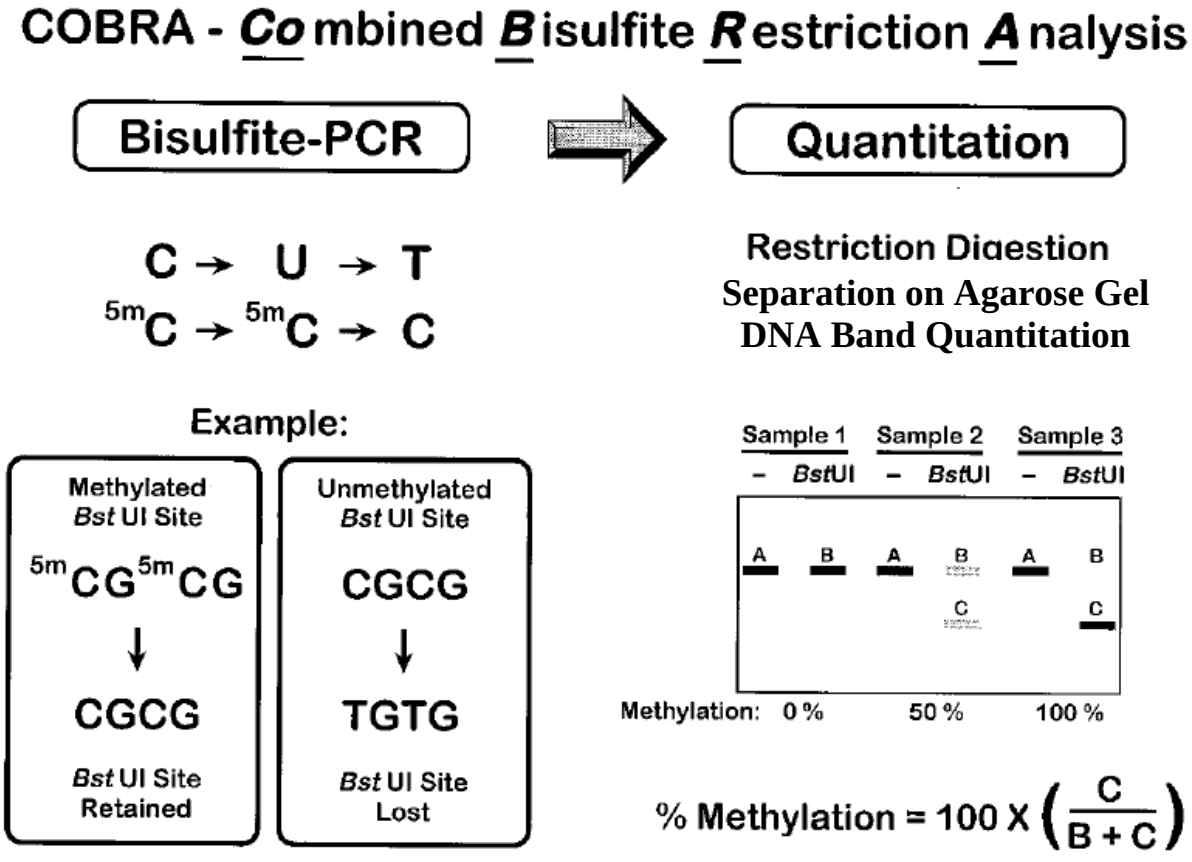


Figure 3 - Flowchart of COBRA using the restriction enzyme Bst U1 in place of Taq1. Adapted from Xiong, Z., & Laird, P [20]

We also used bisulfite sequencing of the region harboring the CpG island to get detailed information about the CpG island methylation pattern. Because COBRA uses restriction enzymes that are sequence specific, we could only test the methylation status of certain CpGs within the island that were present in the sequence recognized by a methylation-sensitive restriction enzyme. Using bisulfite sequencing, it is possible to analyze the entire sequence and therefore could obtain more information compared to COBRA.

Bisulfite sequencing uses the same principle as COBRA. Bisulfite-converted DNA from a pool of cells is amplified and the PCR product is cloned into a TA-cloning vector to produce plasmids. These plasmids are propagated in the bacteria *Esherichia coli* (E. coli). After isolation of the plasmids, primers that are specific to the vector sequence are used to sequence the PCR product within the plasmid from each clone. Using multiple clones and sequencing data, we were able to determine the frequency of methylation of each CpG dinucleotide in the TAM sensitive cells compared to the TAM resistant cells.

Determine if demethylation of the CpG island in the regulatory region will increase miRNA-221/222 expression.

If we can demonstrate that demethylation of the CpG island can increase miRNA-221/222 expression then this shows there is a direct connection between methylation and expression of miRNA-221/222.

We treated TAM sensitive MCF-7 breast cancer cells with different concentrations of decitabine, a clinically approved demethylating agent, at different concentrations by adding decitabine directly to the media and for different lengths of time. We then harvested the cells at desired time points and to isolate total RNA. We then analyzed miRNA-221 and miRNA-222

expression levels using Real-Time RT-PCR to determine if the demethylating agent affected miRNA-221/222 expression.

Materials and Methods

Cell Culture and Tissue Procurement

Tamoxifen-sensitive MCF-7 cells and OHTR cells were obtained from Dr. Kenneth P. Nephew (Indiana University) and maintained as described [19]. Before all experiments, MCF-7 and TAM-resistant MCF-7 cells were cultured in the growth media (MEM with 2mmol/L L-glutamine, 0.1mmol/L nonessential amino acids, 50 units/mL penicillin, 50 mg/ml streptomycin, 6 ng/ml insulin and 10% FBS). Cells were plated to 70% confluency and allowed to grow for 24 hrs before adding TAM (4-hydroxytamoxifen, Sigma) or 40% and allowed to grow for 12 hrs before adding decitabine (Sigma).

TaqMan Reverse Transcription (RT)-PCR for miRNA Quantification

Total RNA was isolated from cell lines with Trizol™ (Invitrogen) according to manufacturer's protocol. MiRNA quantification was carried out by reverse transcribing total RNA using Taqman™ microRNA reverse transcription kit and subjecting it to real-time PCR using TaqMan™ MicroRNA Assay kit (Applied Biosystems). Reactions were performed using Stratagene Mx3000 instrument in triplicate. MiRNA expression was normalized to snRNA RNU6B, RNU44 and/or RNU48. The real-time data was analyzed using a $\Delta\Delta$Ct calculation. A p-value of less than 0.05, when considering treated vs. untreated cells, was considered significant.

Luciferase Assay

Luciferase plasmids pGL3-basic and pRLtk were purchased from Promega. Various regions of the miRNA-221/222 promoter were inserted into the multiple cloning site of the pGL3-basic plasmid using conventional molecular cloning techniques. Plasmid DNA was

purified by QIAprep Midiprep Kit (Qiagen). MCF-7 and OHTR cells were maintained in culture as described above and transfection of plasmids was carried out using Lipofectamine2000 (Invitrogen) according to manufacturer's protocol. MCF-7 and OHTR cells were plated at $1x10^5$ cells per well in a 24-well plate 24 hr prior to transfection. For each transfection reaction, the control plasmid (pRLtk) containing *Renilla* luciferase driven by Thymidylate kinase (tk) promoter was mixed with the firefly luciferase construct at a 1:50 molar ratio to allow for accurate normalization. The transfection mixture was added to the cells and allowed to incubate for 5 hr at 37°C before replacing with fresh media. After allowing the cells to grow for 12 hr, they were treated with different concentrations of TAM. The cells were harvested 48 hr after transfection for the luciferase assay. To measure activity of the cloned promoter, firefly and *Renilla* luciferase activity was measured sequentially from a single cell lysate on a TriStar LB941 (Berthold Technologies) by using the dual luciferase assay system (Promega) according to manufacturer's protocol. The results are expressed as a ratio of firefly luciferase to *Renilla* luciferase.

Northern Blot

Total RNA was isolated from different cell lines as described above. Thirty micrograms total RNA was separated by 15% polyacrylamide:bis gel electrophoresis and transferred to a nylon membrane. The RNA was UV-crosslinked to the membrane and then baked at 80°C for 30 min. The membrane was then pre-hybridized for 1 hr at 42°C before hybridizing with [γ-^{32}P]dCTP-labeled anti-miRNA-222 or miRNA-221 probe overnight at 42°C. Post-hybridization, membranes were washed twice with non-stringent buffer and once with a stringent buffer. The blots were subjected to autoradiography to determine expression of miRNA-221/222.

RT PCR for Primary Transcript

Total RNA was isolated as described above and reverse transcribed using Multiscribe Reverse Transcriptase (Invitrogen) and a reverse primer specific for the miRNA-221/222 primary transcript. The cDNA was the subjected to PCR using conventional techniques. The reverse primer used for PCR was identical to the primer used for transcription and paired with a forward primer specific for miRNA-221/222. PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. All sequencing was performed by The Ohio State University's Nucleic Acid Shared Resource Center per their protocol.

COBRA

Genomic DNA was isolated from MCF-7 cell line, cells that had been treated with TAM for 3 months with the highest concentration being 55nM (LT55), and OHTR cell lines and bisulfite converted. 26ng of converted DNA from each cell line was used for hot-start PCR in order to amplify the CpG island genomic region. Hot-start PCR uses the same composition as a regular RT PCR reaction, but the polymerase and reaction buffer are added to the other reagents after they have already been heated to 94 degrees centigrade. This product was purified using Qiagen gel purification kit and re-amplified with the same primer set. The second product was digested with TSP5091 or Taq1 according to manufacturer's protocol (NEB), the products were separated on a 2% agarose gel and stained with ethidium bromide for viewing in UV light. The DNA Bands were quantified using Kodak software for gel quantification. Full detailed protocol can be found in article by Xiong, Z., & Laird, P [20].

Bisulfite Sequencing

Genomic, bisulfite treated DNA from MCF-7 and OHTR cells was amplified using primers specific for the CpG island spanning the miRNA-221/222 promoter (as above). The

product from this PCR was cloned in the pDrive cloning vector according to Qiagen's PCR Cloning Kit. Blue/white screening was used to select clones positive for PCR product [28]. These clones were expanded in E.coli and plasmid was isolated using QIAprep MidiPrep Plasmid Isolation Kit (Qiagen). These plasmids were then sequenced. All sequencing was performed by The Ohio State University's Nucleic Acid Shared Resource Center per their protocol.

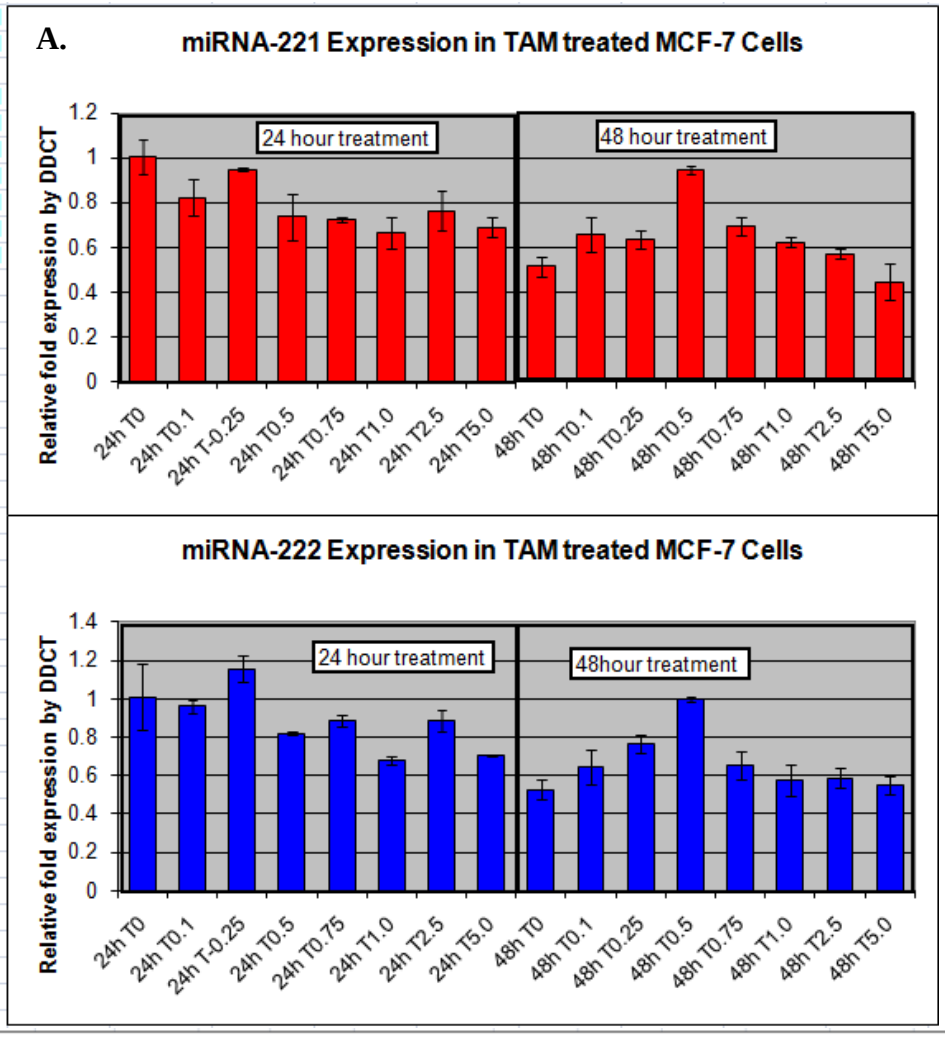
Results

Tamoxifen induces miRNA-221 and miRNA-222 in MCF-7 cells over a long term treatment. Our previous study found that miRNA-221/222 were dramatically upregulated in a TAM-resistant cell line that was derived from a TAM-sensitive cell line by treating the parental cells with TAM over several months. This led us to believe that TAM could induce miRNA-221/222 and two systems were tested to examine if this induction occurred. First, we treated TAM-sensitive MCF-7 cells with different concentrations of TAM for 24 and 48 hours, isolated total RNA from these cells and tested miRNA-221 and miRNA-222 expression using Real-Time RT-PCR. We found a dramatic dose dependent increase in miRNA-221/222 expression in response to tamoxifen in the first experiment. However, this experiment was repeated several times with varying concentrations of TAM and varying treatment times, but the results of the initial test could not be reproduced. We therefore concluded that acute TAM treatment does not induce miRNA-221/222 (Figure 4a,b,c). This may be because the cells need longer time to develop alternate mechanism to survive in the absence of estrogen. Developing resistance over a long period of time is more representative of the clinical development of resistance in breast cancer patients where it may take years to develop TAM resistance.

Next, we treated TAM-sensitive cells with low doses of TAM for long periods of time to reveal if there was a gradual increase in miRNA-221/222 expression. Sensitive cells were treated with 15 nM TAM and gradually, over a period of 5 months, the TAM concentration was increased in increments of 5-10 nM until the cells reached a treatment concentration of 100 nM TAM. Before each increase in TAM concentration, a fraction of the cells were harvested and RNA isolated. Real-Time RT-PCR analysis of the isolated total RNA from these cells revealed that there is a gradual increase in miRNA-221/222 expression in response to tamoxifen in MCF-7 cells (Figure 4d). The media concentration of TAM at time of harvesting and duration of treatment is noted on the graph. This long-term treatment experiment was repeated two times, independent of each other, with very similar results.

With this experiment, we have shown that tamoxifen can induce miRNA-221/222 in MCF-7 breast cancer cells. Using this model we planned to determine the mechanism of upregulation of miRNA-221/222. We next examined all three types of regulation reported for miRNA: transcriptional, post-transcriptional and epigenetic regulation.

Figure 4


A.
miRNA-221 Expression in TAM treated MCF-7 Cells
Relative fold expression by DDCT
24 hour treatment
48 hour treatment
24h T0
24h T0.1
24h T-0.25
24h T0.5
24h T0.75
24h T1.0
24h T2.5
24h T5.0
48h T0
48h T0.1
48h T0.25
48h T0.5
48h T0.75
48h T1.0
48h T2.5
48h T5.0
miRNA-222 Expression in TAM treated MCF-7 Cells
Relative fold expression by DDCT
24 hour treatment
48hour treatment

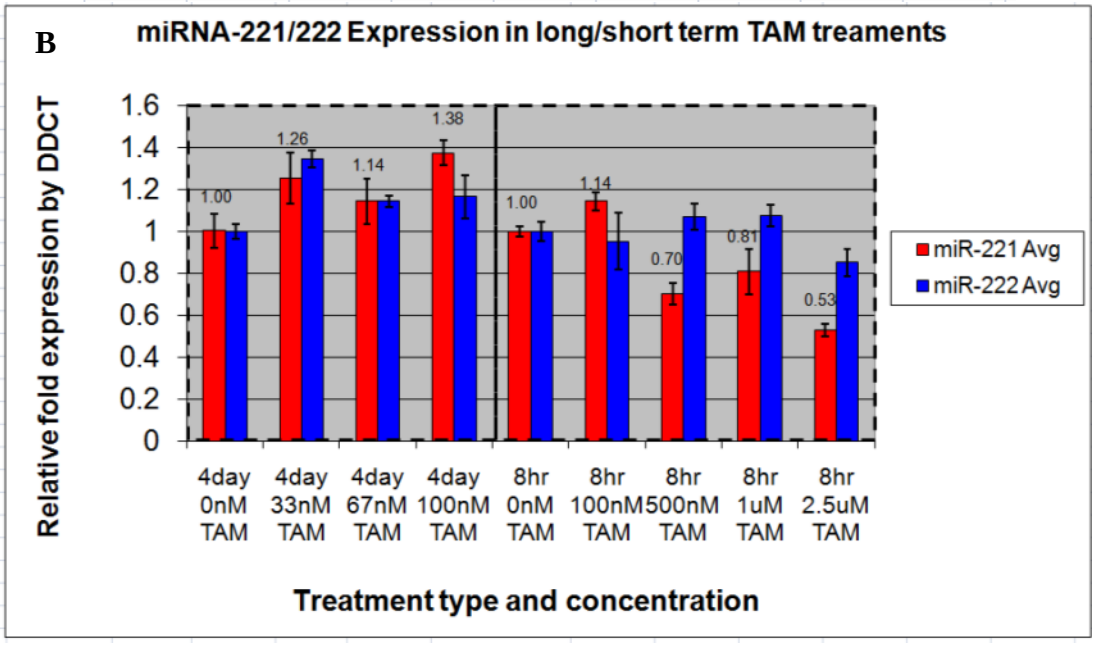

B
miRNA-221/222 Expression in long/short term TAM treaments
Relative fold expression by DDCT
1.00
1.26
1.14
1.38
1.00
1.14
0.70
0.81
0.53
4day 0nM TAM
4day 33nM TAM
4day 67nM TAM
4day 100nM TAM
8hr 0nM TAM
8hr 100nM TAM
8hr 500nM TAM
8hr 1uM TAM
8hr 2.5uM TAM
miR-221 Avg
miR-222 Avg
Treatment type and concentration

Figure 4C.

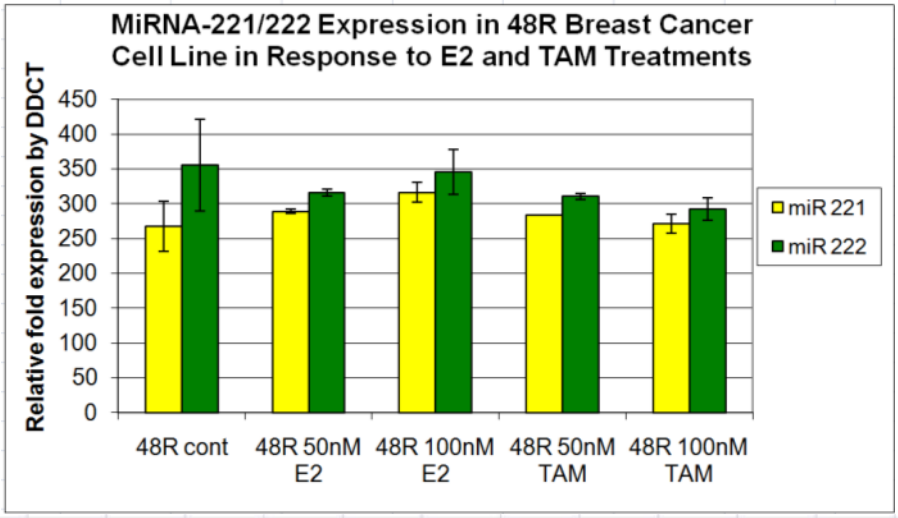


Figure 4D.

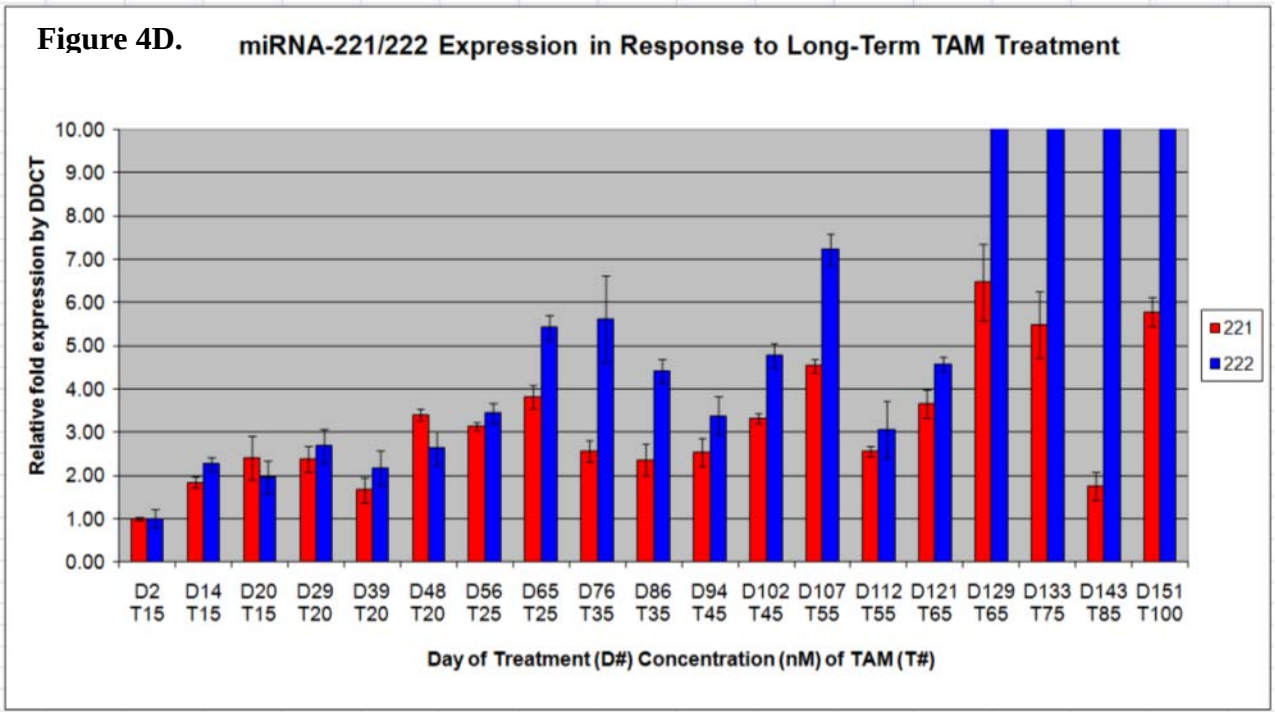


Figure 4.– A) Real-time PCR results of MCF-7 breast cancer cells treated with tamoxifen at varying concentration for either 24 or 48 hrs. The top graph is miRNA-221 expression and the bottom is miRNA-222 expression. T# represents the tamoxifen concentration in μM. *B)* Real-Time PCR results of MCF-7 breast cancer cells treated with TAM for either 4 days (4d) or 8 hrs (8hr). *C)* Real-time PCR results in 48R breast cancer cells of miRNA-221/222 expression after treatment with estrogen (E2) or TAM. *D)* Real-time PCR results of miRNA-221/222 expression of long term TAM treatment on MCF-7 cells. Day of Treatment (D#) is the time from initial treatment and (T#) is concentration of TAM in nM.

Luciferase assay reveals no significant effect of tamoxifen on the promoter activity. In order to determine if miRNA-221 /222 is transcriptionally regulated and the upstream promoter region regulating this expression, we studied the miRNA-221/222 promoter using a luciferase assay. First, we designed luciferase reporter plasmids where different regions of the miRNA-221/222 promoter was cloned (Figure 5a). We then transfected the various plasmids into MCF-7 cells. As the luciferase coding region in these plasmids is under control of different regions of the miRNA-221/222 promoter, the amount of luciferase gene transcribed is directly related to the activity of the promoter segment. We first tested baseline activity of each promoter region in MCF-7 and OHTR cell lines that were not treated TAM. This revealed that the most important region of the promoter for active transcription was 225bp upstream of the transcription start site of the miRNA-221/222 transcript (Figure 5b,c).

In order to determine the promoter region that is responsive to TAM treatment, we treated cells with varying concentrations of TAM after transfection with the promoter/luciferase plasmids. Surprisingly, there was no significant change in the promoter activity with TAM treatment, irrespective of the reporter plasmid used for the study (Figure 5d). This experiment was repeated with various concentrations of TAM and several other transfection and treatment conditions, but no significant results were obtained. These results indicate that TAM does not increase transcription in the miRNA-221/222 promoter region that was analyzed.

Figure 5

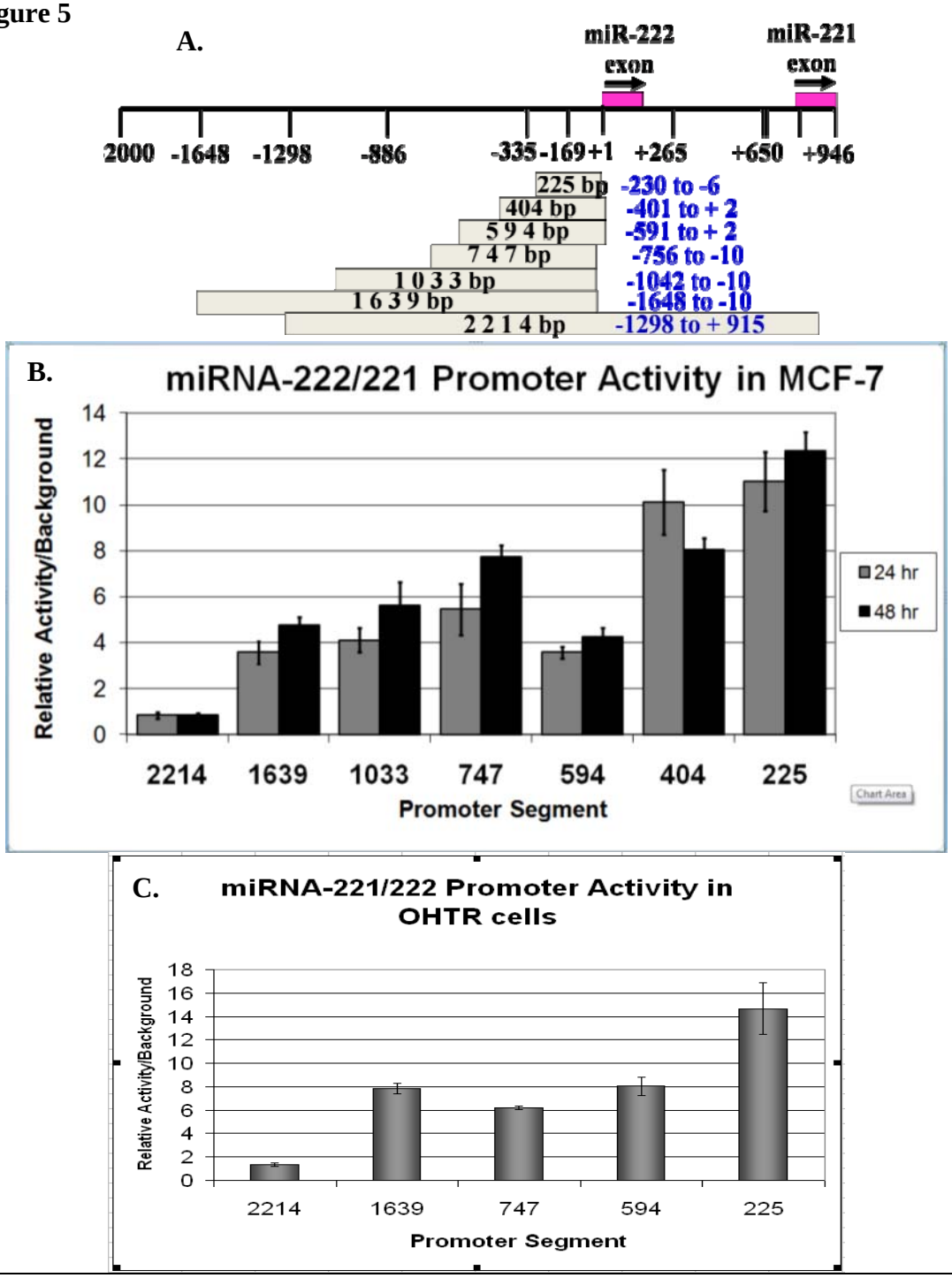
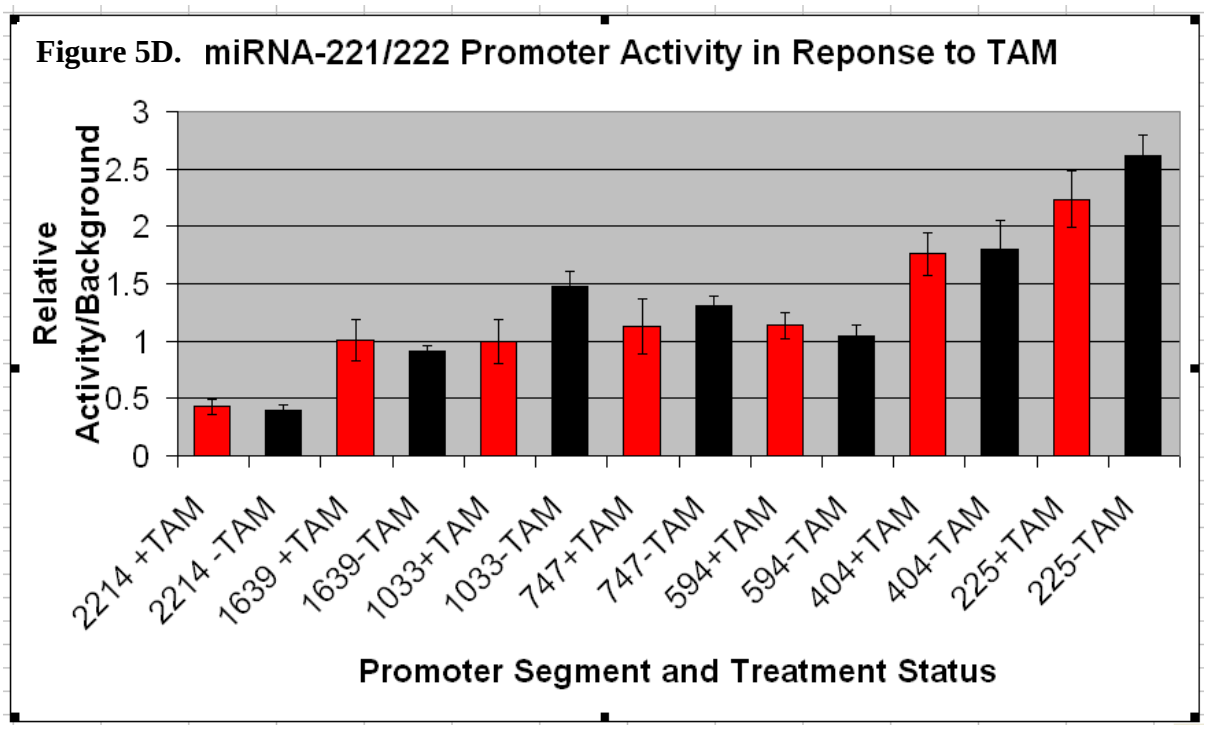


Figure 5 - A) Schematic of promoters in the luciferase assay. miRNA -221/222 are transcribed in direction indicated by arrows. Transcription start site of miRNA-222 gene is indicated by +1 site. Promoter segment size is indicated inside boxes and location, relative to the +1 site, is given by the numbers adjacent to each box. *B)* Baseline activity of miRNA-221/222 promoter by luciferase expression in MCF-7 cells and *C)* OHTR cells. OHTR cells were analyzed 48 hrs after transfection while MCF-7 cells were analyzed 24 or 48 hrs, as indicated, after transfection. *D)* miRNA-221/222 promoter segment activity in response to TAM treatment (2.5uM TAM for 48 hrs). All data are averages of triplicate wells and duplicate luminometer readings.



Post-transcriptional regulation studies prove unsuccessful. As there are several levels of miRNA processing after the intitial transcription of miRNA-221/222, and these processing steps have been reported to have regulatory ability, we next investigated if this post-transcriptional regulation was controlling miRNA-221/222 expression. In theory, we could compare the level of expression of the primary transcript, the precursor and the mature miRNA in TAM treated cells and untreated cells. If there was regulation occurring at the processing level due to TAM, we should see an unproportional increase in the mature miRNA compared to the primary transcript or precursor in the TAM treated cells. For example, if TAM caused an increase in the processing of the primary transcript, and there was no increase in transcription, then we would find a lower expression level of the miRNA-221/222 primary transcript in the TAM treated MCF-7 cells. This hypothetical example would cause an increase in the mature miRNA in the TAM treated cells, which is what we find in reality in our long-term TAM treated cells.

Our initial attempt at detecting the miRNA-221/222 primary transcript, the miRNA precursor and the mature miRNA was to use northern blotting. To test our detection capabilities, we isolated total RNA from MCF-7 and MCF-7 cells overexpressing miRNA-221/222 (These cells express about 12 fold higher levels of miRNA-221/222 than regular MCF-7 cells). We ran the total RNA on a gel and transferred the RNA to a membrane. We then probed this membrane with ^{32}P-labeled probes specific for a sequence in the miRNA-221/222 transcripts. After autoradiography, we were unable to detect the primary transcript in cells overexpressing miRNA-221/222 and were scarcely able to see the precursor and mature miRNA (Figure 6). We were unable to detect the expression of miRNA-221/222 at any level in the MCF-7 cells. Multiple optimization attempts did not significantly improve our detection ability. We concluded that the expression level of miRNA-221/222 is not high enough in our breast cancer cell line to use northern blot technology for its detection.

Figure 6

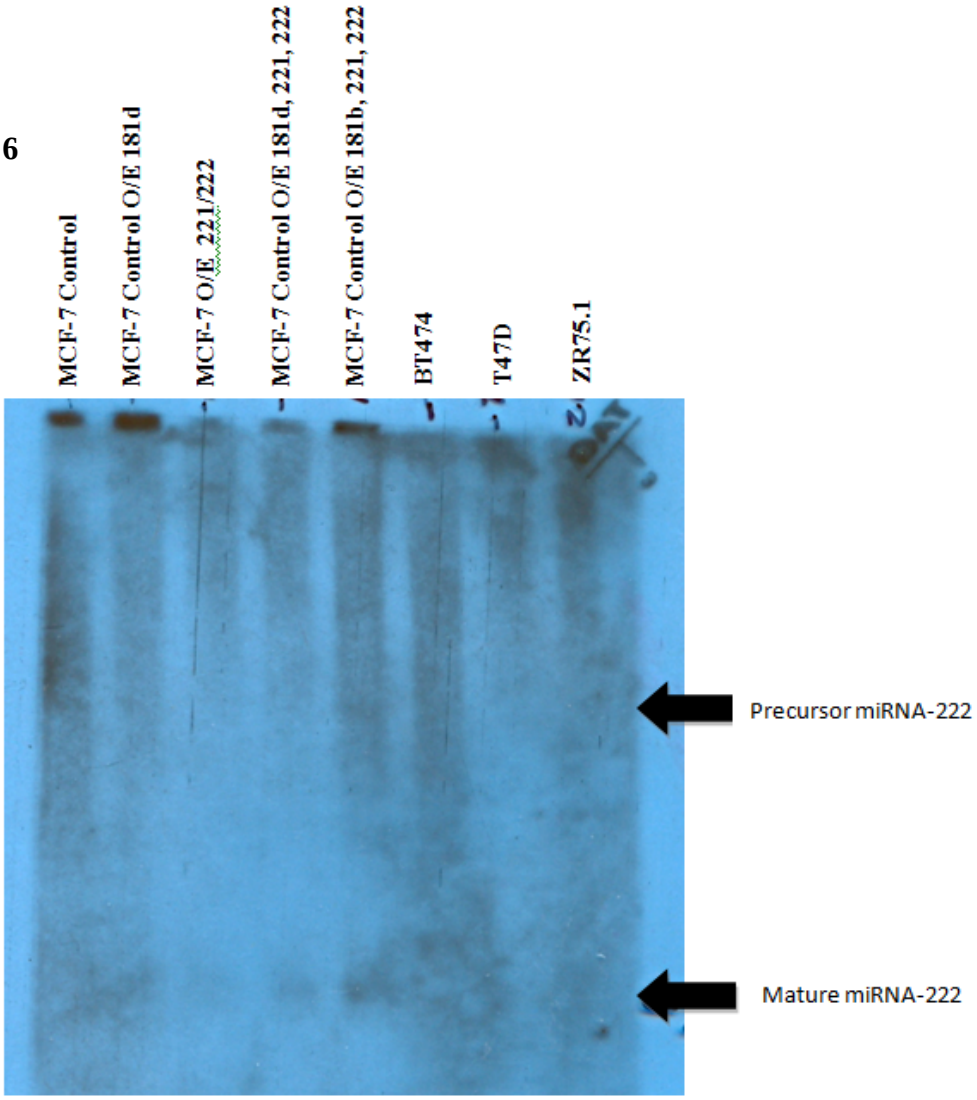


Figure 6 - Northern Blot analysis of MCF-7 cells and MCF-7 derivatives that overexpressed (O/E) miRNA-221/222. The three wells farthest to the right contain total RNA from three different breast cancer cells lines. Probe was specifically designed for miRNA-222 and Mature and precursor miRNA-222 is identified by arrows.

Our next attempt was to detect the primary transcript using PCR technology, as this requires less miRNA-221/222 transcript for detection. The strategy for this technique is to enrich the cDNA with miRNA-221/222 primary transcript by using a reverse primer specific for miRNA-221/222 instead of a random hexamer primer during reverse transcription of the total

RNA. After enriching the miRNA-221/222 cDNA, we amplified the primary transcript using PCR and the same reverse primer used in reverse transcription along with its corresponding forward primer was used (Figure 7a). Although our MCF-7 genomic DNA control gave robust PCR product, we could not detect the primary transcript in the enriched cDNA. We attempted this experiment with several primer sets, but could not detect the primary transcript (Figure 7b,c,d). We did, however, detect products that were smaller than predicted, but subsequent sequencing revealed these to be nonspecific products. These results may be due to unknown processing in the miRNA-221/222 primary transcript, as this is a clustered miRNA transcript, which leads the predicted product being truncated and undetectable by the primers we are using. Finally, and most likely, the primary transcript may be processed immediately and efficiently by Drosha and therefore the primary transcript expressed in the cell was not detectable.

Figure 7

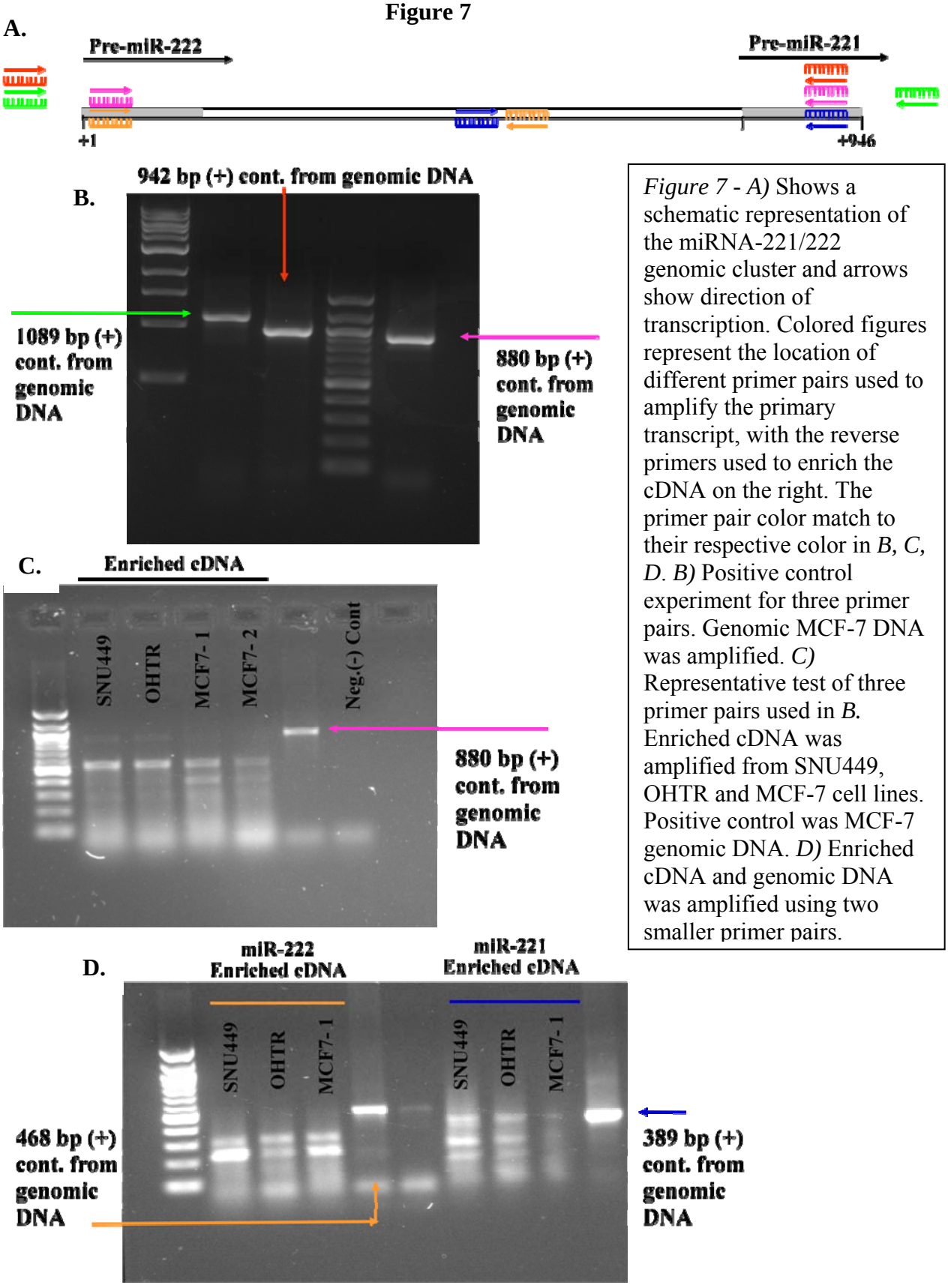


Figure 7 - A) Shows a schematic representation of the miRNA-221/222 genomic cluster and arrows show direction of transcription. Colored figures represent the location of different primer pairs used to amplify the primary transcript, with the reverse primers used to enrich the cDNA on the right. The primer pair color match to their respective color in *B, C, D. B)* Positive control experiment for three primer pairs. Genomic MCF-7 DNA was amplified. *C)* Representative test of three primer pairs used in *B.* Enriched cDNA was amplified from SNU449, OHTR and MCF-7 cell lines. Positive control was MCF-7 genomic DNA. *D)* Enriched cDNA and genomic DNA was amplified using two smaller primer pairs.

These findings indicate it was not possible to conclude if TAM had any effect on miRNA-221/222 processing using these techniques. It may be possible to detect the primary transcript and miRNA precursor if the processing enzymes were blocked in a stepwise fashion. This would allow the transcripts to be produced without being processed and accumulate to detectable levels in the cell. This experiment would need to be completed before we can deduce if post-transcriptional regulation of miRNA-221/222 plays a role in tamoxifen resistance.

CpG island methylation shows significant demethylation in select CpG. We discovered a CpG island in the miRNA-221/222 promoter about 8kb upstream of the genomic miRNA-221/222 region through MethPrimer, an online software application (http://www.urogene.org/methprimer/index1.html). We proceeded to analyze the CpG island through COBRA analysis. Our COBRA analysis was confined to one CpG out of the 11 CpGs in the island due to lack of suitable restriction enzyme site. It is still useful to measure the methylation status of one CpG within an island because the methylation status of one CpG can closely represents the methylation status of all CpGs within the island. Our analysis of a CpG in the middle of the island revealed a significant decrease in methylation in the OHTR cell line. This trend of demethylation was also seen in cells that had been treated with TAM over a long time period, which at the time of harvest were being treated with 55nM TAM (LT55) (Figure 8a,b). This is to be expected because the OHTR cell line was created by treating MCF-7 cells with TAM over a long time course. If OHTR cells have less methylation then it would be expected that cells treated with TAM gradually lose their methylation over time. This demethylation is in concordance with the increase in miRNA-221/222 expression in OHTR cells as DNA methylation generally represses transcription.

Next, in order to get a more complete view of the methylation status of the entire CpG island, we conducted bisulfite sequencing of the CpG island in MCF-7 and OHTR cells. We were able to sequence nearly every CpG base pair in the island. Eighteen clones from each cell line were sequenced and the complete sequences were analyzed. We found that there was no significant change in the methylation of the CpGs except for in CpG #8 (Figure 8c). Interestingly, this was also the specific CpG that we analyzed via COBRA analysis. Because methylation of a single CpG can influence transcription due to disruption of transcription factor binding, we immediately looked into the sequence surrounding this CpG for transcription factor binding sites. We used TESS software from the University of Pennsylvania (http://www.cbil.upenn.edu/cgi-bin/tess/tess) to analyze the genomic sequence according to website instructions. This analysis revealed no promising binding sites that could explain the increased miRNA-221/222 expression in our system. As only 1/11 CpGs in the island had differential methylation, it is very improbable that repression by collective CpG methylation was the cause of the different miRNA-221/222 expression in MCF-7 versus OHTR cells.

Figure 8

A.

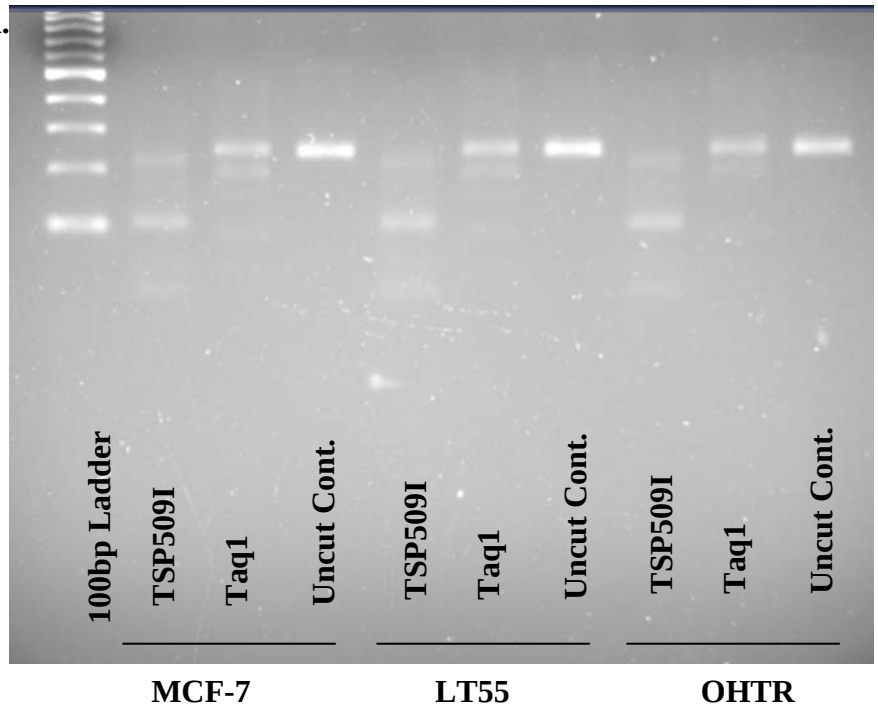


Figure 8B.

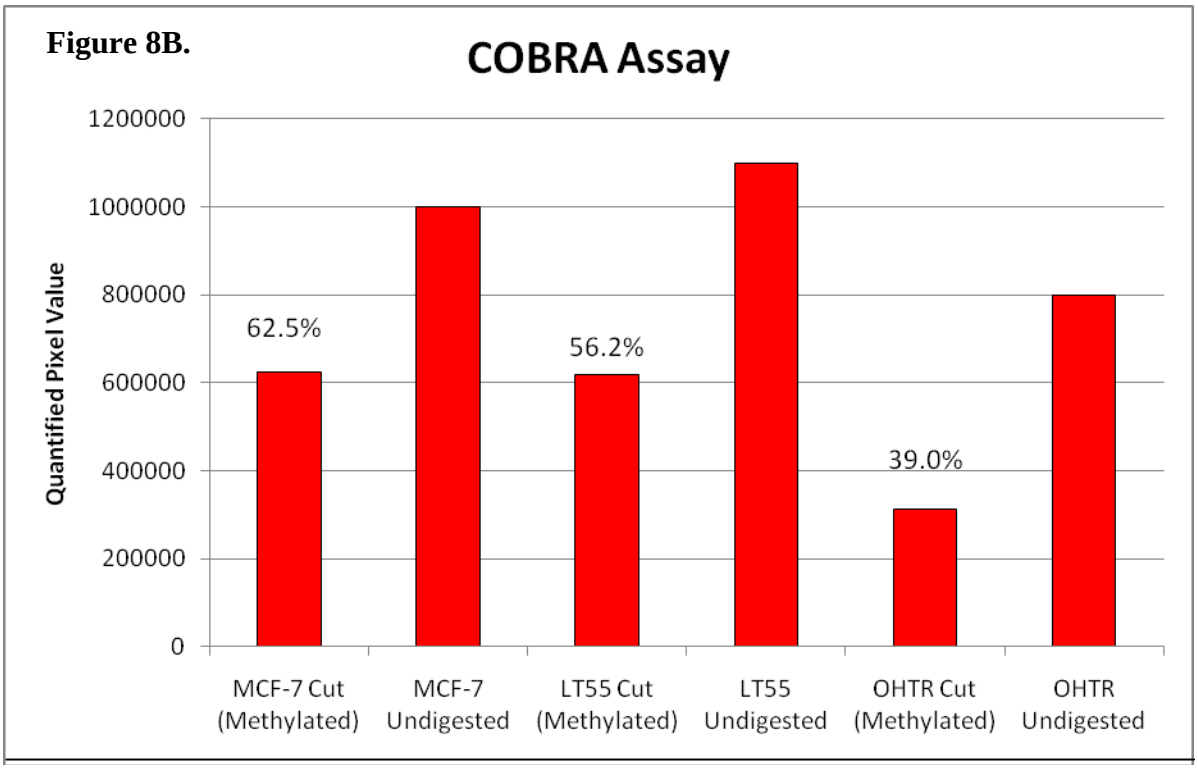
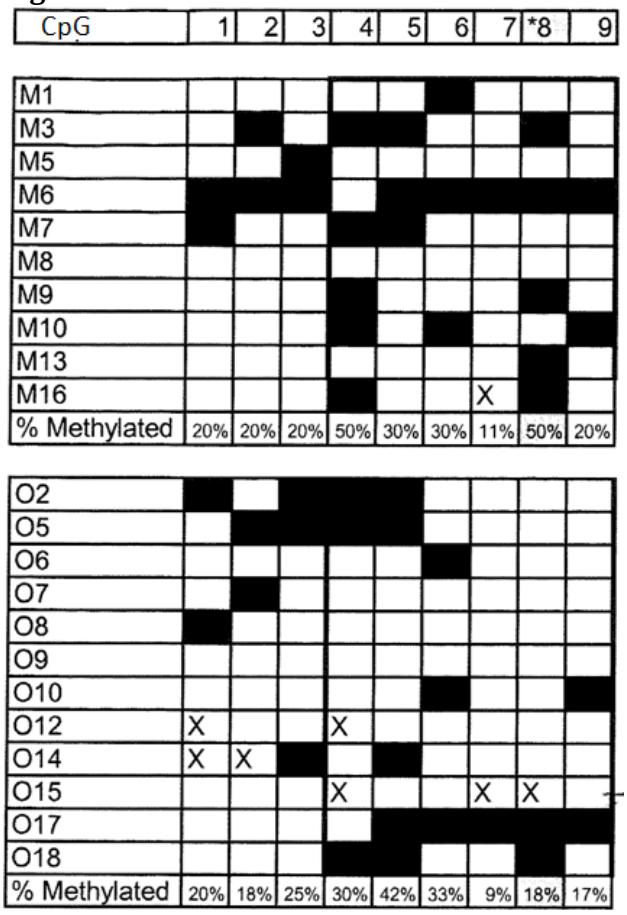


Figure 8C.



CpG	1	2	3	4	5	6	7	*8	9
M1						■			
M3		■		■	■			■	
M5			■						
M6	■	■	■		■	■	■	■	■
M7	■			■	■				
M8									
M9				■				■	
M10				■		■			■
M13								■	
M16				■			X	■	
% Methylated	20%	20%	20%	50%	30%	30%	11%	50%	20%

O2	■		■	■	■				
O5		■	■	■	■				
O6						■			
O7		■							
O8	■								
O9									
O10						■			■
O12	X			X					
O14	X	X	■		■				
O15				X			X	X	
O17					■	■	■	■	■
O18				■	■			■	
% Methylated	20%	18%	25%	30%	42%	33%	9%	18%	17%

Figure 8 - A) Results from COBRA on 2.0% agarose gel. The enzyme used to digest the CpG island amplified product is listed below each well. Product cut with TSP509I determines if full bisulfite conversion took place. Taq1 was used to determine if the specific CpG was methylated. If it the product is cut it represents bisulfite conversion or methylation of the CpG. *B)* Quantification of COBRA gel. Percentage above bars equals the percentage of methylation of the CpG in that cell line. Y-axis value is assigned by Kodak gel quantification software. *C)* Bisulfite sequencing analysis of the CpG island in MCF-7 (M# - top) and OHTR (O# - bottom) cell lines. Filled black boxes represent methylation, while white represent no methylation. Percent of the clones which had methylation at the specific CpG in the island is listed below each table.

Treatment of MCF-7 with decitabine and TAM reveals small increases in miRNA-221/222.

In another attempt to link DNA methylation with the regulation of miRNA-221/222 expression, we used a demethylating agent, decitabine, to demethylate the CpG island on the miRNA-221/222 promoter. If demethylation of the promoter led to an increase in miRNA221/222 expression, then promoter methylation could be linked to miRNA-221/222 regulation. In initial experiments, MCF-7 cells were treated with decitabine for about a week and no induction of miRNA-221/222 was seen (data not shown). We also tried to combine TSA and decitabine, as TSA is a potent inhibitor of histone deacetylase and has been shown reduce DNA methylation leading to re-expression of silenced genes [26], but we again saw no significant induction in expression (data not shown).

In this short term treatment setting, we also investigated the effect of TAM and decitabine in combination on miRNA-221/222 expression. The rational was that while repression by DNA methylation may be partially responsible for the regulation, transcriptional activation by TAM may also play a role. It is highly likely that demethylation or TAM treatment alone is not sufficient to increase miRNA-221/222 expression, but together they might have a synergistic effect in activating the promoter and may prove to be the key to miRNA-221/222 regulation. We have not observed significant alteration in miRNA-221/222 expression after short term decitabine/TAM treatments, so we designed a long term decitabine/TAM treatment regimen to try to induce miRNA-221/222 expression. We continuously treated MCF-7 cells with decitabine over 20 days, escalating the dose in some cells at different time points while also maintaining cells with initial doses and untreated control cells. During the experiment, we treated decitabine pre-treated cells with TAM for a short time period before harvesting the cells to collect miRNA-221/222 expression data. The various treatment combinations during this experiment produced insignificant inductions in expression, except for the last time point at 20 days (Figure 9). During this time point, the cells were treated with TAM for 4 days instead of 48 hrs. We know from previous experiments that it may take several weeks to get significant increases in expression with TAM alone, so the finding that longer TAM treatment leads to greater inductions is not surprising. While this experiment has not been reproduced, it does give some indication that the methylation status of the cells may play a role in miRNA-221/222 regulation. This finding needs to be further explored.

Figure 9

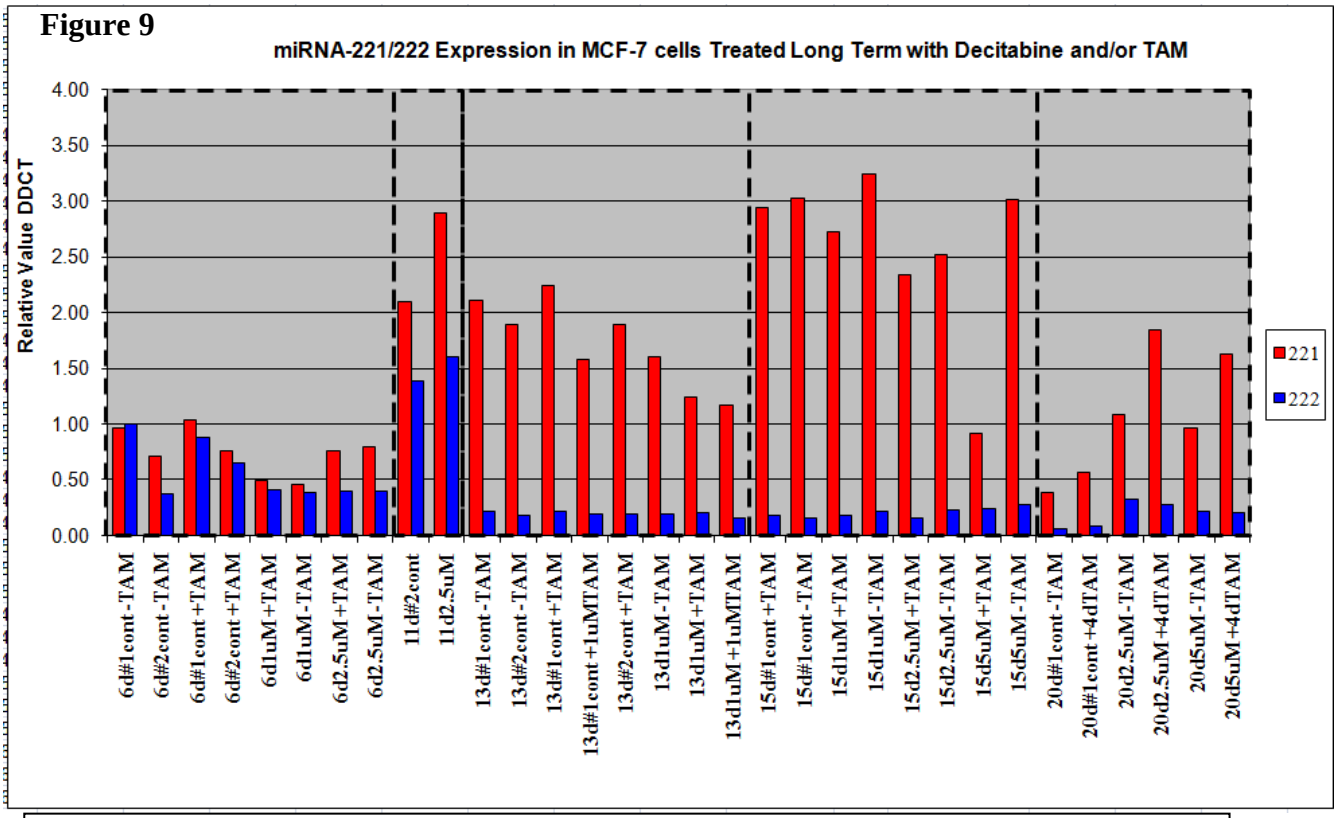


Figure 9 - Real-time PCR analysis of miRNA-221/222 expression in MCF-7 cells treated with decitabine and/or TAM over the course of 20 days. Expression was normalized to RNU44/RNU48. TAM concentration is 100nM unless otherwise specified on graph and TAM treatment was for 48 hrs before harvesting unless specified on graph. The #d at the beginning of each sample label is the number of days cells have been treated with decitabine and concentration of decitabine at time of harvest directly follows the number of days. Sample labels that have cont immediately following day number have not been treated with decitabine.

Discussion

In this study, we have attempted to elucidate the mechanism by which miRNA-221/222 are upregulated in TAM resistant breast cancer. Currently, three different mechanisms that can regulate miRNA expression have been reported. These are transcriptional regulation, post-transcriptional regulation and epigenetic regulation by DNA methylation. We believe that miRNA-221/222 are regulated by one of these mechanisms or a combination of these

mechanisms. We tested for all three types of regulation in our system, but were unsuccessful in pinpointing the regulation of miRNA-221/222.

However, we were able to demonstrate that long term exposure to TAM does induce miRNA-221/222 expression. The increased expression of these miRNAs then causes resistance. Essentially, we have shown in a breast cancer cell line that tamoxifen can be the cause of its own acquired resistance and this may also be a cause of resistance in women with breast cancer. Our luciferase assay results also confirm the results that were found in a recently published paper [18], that transcriptional activity of the miRNA-221/222 promoter was highest in the regions most proximal to the miRNA-221/222 transcription start site. This finding is inconsistent with the paper that determined by bioinformatic analysis that the TSS of miRNA-221/222 was 15kb upstream of the genomic region [17]. Finally, we have shown that there is a different methylation pattern TAM resistant cells compared to TAM sensitive cells. While this difference is not overwhelming, it is interesting and may be enough to cause some regulation of expression of miRNA-221/222. This is also supported by limited demethylation data that showed slight increases in expression in response to long term treatment of MCF-7 cells with decitabine and TAM.

Work will continue on this project because the elucidation of the regulation of miRNA-221/222 would be a very significant advancement. It is important because the upregulation of the two miRNAs is sufficient to induce TAM resistance in a TAM sensitive breast cancer cell line and may be a mechanism for this resistance in human breast tumors. As a large proportion of women who are treated with this important anti-cancer drug are resistant to it, exploring the causes for their resistance is crucial for the patients. By discovering the mechanism of tamoxifen resistance in patients, we may be able to develop therapeutic drugs that target molecules in this

pathway and restore sensitivity to tamoxifen. This would allow more patients to use this powerful drug that has relatively few side effects.

Revealing the regulation of miRNA-221/222 may also have large implications in many areas outside of TAM resistant breast cancer as it has been reported to be involved in many cancers. In fact, within the last year there have been reports published that indicate a role for miRNA-221/222 in urothelial carcinoma [21], gastric cancer [22], primary effusion lymphoma and Kaposi sarcoma [23], glioma [24], and interestingly in castration-resistant prostate cancer [25]. Also, data from Dr. Kenneth Nephew's lab at Indiana University suggests that miRNA-221/222 play a role in fulvestrant resistant breast cancer. It is obvious that these two microRNAs play a very important role in a wide variety of cancers and drug resistance. It is possible that by discovering the mechanism that regulates these two powerful miRNAs in tamoxifen resistant breast cancer, we will also reveal its regulation in many other diseases. This will improve our knowledge about the mechanisms of these diseases and may lead to advancements in treatments for patients.

Bibliography

1) Early Breast Cancer Trialists' Collaborative Group (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *The Lancet*, *365*(9472), 1687-717.
2) Filipowicz, W., Bhattacharyya, S., & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature Reviews Genetics*, *9*(2), 102-14.
3) Thomson, J., Newman, M., Parker, J., Morin-Kensicki, E., Wright, T., & Hammond, S. (2006). Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes & Development*, *20*(16), 2202-7.
4) Croce, C. (2008). Oncogenes and cancer. *New England journal of medicine*, *358*(5), 502-11.
5) Iorio, M., Ferracin, M., Liu, C., Veronese, A., Spizzo, R., Sabbioni, S. et al. (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Research*, *65*(16), 7065-70.

6) Miller, T., Ghoshal, K., Ramaswamy, B., Roy, S., Datta, J., Shapiro, C. et al. (2008). MicroRNA-221/222 Confers Tamoxifen Resistance in Breast Cancer by Targeting p27Kip1. *The Journal of Biological Chemistry*, *283*(44), 29897-903.
7) Zhao, J., Lin, J., Yang, H., Kong, W., He, L., Ma, X. et al. (2008). MicroRNA-221/222 Negatively Regulates Estrogen Receptor{alpha} and Is Associated with Tamoxifen Resistance in Breast Cancer. *The Journal of biological chemistry*, *283*(45), 31079-86.
8) Si, M., Zhu, S., Wu, H., Lu, Z., Wu, F., & Mo, Y. (2007). miR-21-mediated tumor growth. *Oncogene*, *26*(19), 2799-803.
9) Meng, F., Henson, R., Lang, M., Wehbe, H., Maheshwari, S., Mendell, J. et al. (2006). Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology*, *130*(7), 2113-29.
10) Yang, H., Kong, W., He, L., Zhao, J., O'Donnell, J., Wang, J. et al. (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Research*, *68*(2), 425-33.
11) Datta, J., Kutay, H., Nasser, M., Nuovo, G., Wang, B., Majumder, S. et al. (2008). Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. *Cancer Research*, *68*(13), 5049-58.
12) Wang, B., Hsu, S., Majumder, S., Kutay, H., Huang, W., Jacob, S. et al. (2010). TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. *Oncogene*, *29*(12), 1787-97.
13) Felicetti, F., Errico, M., Bottero, L., Segnalini, P., Stoppacciaro, A., Biffoni, M. et al. (2008). The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. *Cancer Research*, *68*(8), 2745-54.
14) Ciafr, ., è, S., Galardi, S., Mangiola, A., Ferracin, M., Liu, C. et al. (2005). Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochemical and Biophysical Research Communications*, *334*(4), 1351-8.
15) Pallante, P., Visone, R., Ferracin, M., Ferraro, A., Berlingieri, M., Troncone, G. et al. (2006). MicroRNA deregulation in human thyroid papillary carcinomas. *Endocrine-Related Cancer**, *13*(2), 497-508.
16) Lee, E., Gusev, Y., Jiang, J., Nuovo, G., Lerner, M., Frankel, W. et al. (2007). Expression profiling identifies microRNA signature in pancreatic cancer. *International journal of cancer. Journal international du cancer*, *120*(5), 1046-54.
17) Corcoran D, Pandit K, Gordon B, Bhattacharjee A, Kaminski N, Benos P. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. *PLoS ONE*. 2009;4(4):e5279.
18) Di Leva, G., Gasparini, P., Piovan, C., Ngankeu, A., Garofalo, M., Taccioli C, . et al. (2010). MicroRNA Cluster 221-222 and Estrogen Receptor {alpha} Interactions in Breast Cancer.CrossRef DOI Query. *Journal of the National Cancer Institute*, [No Volume/Issue] [No Pagination]
19) Fan, M., Yan, P. S., Hartman-Frey, C., Chen, L., Paik, H., Oyer, S. L., Salisbury, J. D., Cheng, A. S., Li, L., Abbosh, P. H., Huang, T. H., and Nephew, K. P. (2006) *Cancer Res.* 66, 11954–11966
20) Xiong, Z., & Laird, P. (1997). COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Research*, *25*(12), 2532-4.

21) Veerla, S., Lindgren, D., Kvist, A., Frigyesi, A., Staaf, J., Persson, H. et al. (2009). MiRNA expression in urothelial carcinomas: important roles of miR-10a, miR-222, miR-125b, miR-7 and miR-452 for tumor stage and metastasis, and frequent homozygous losses of miR-31. *International journal of cancer. Journal international du cancer*, *124*(9), 2236-42.
22) Kim, Y., Yu, J., Han, T., Park, S., Namkoong, B., Kim, D. et al. (2009). Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. *Nucleic Acids Research*, *37*(5), 1672-81.
23) O'Hara, A., Wang, L., Dezube, B., Harrington WJ Jr, ., Damania, B., & Dittmer, D. (2009). Tumor suppressor microRNAs are underrepresented in primary effusion lymphoma and Kaposi sarcoma. *Blood*, *113*(23), 5938-41.
24) Zhang, C., Kang, C., You, Y., Pu, P., Yang, W., Zhao, P. et al. (2009). Co-suppression of miR-221/222 cluster suppresses human glioma cell growth by targeting p27kip1 in vitro and in vivo. *International Journal of Oncology*, *34*(6), 1653-60.
25) Sun, T., Wang, Q., Balk, S., Brown, M., Lee, G., & Kantoff, P. (2009). The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines. *Cancer Research*, *69*(8), 3356-63.
26) Selker, E. (1998). Trichostatin A causes selective loss of DNA methylation in Neurospora. *Proceedings of the National Academy of Sciences*, *95*(16), 9430-5.
27) http://www.cancer.org/docroot/cri/content/cri_2_4_1x_what_are_the_key_statistics_for_breast_cancer_5.asp. Retrieved 5/12/2010.
28) Hirt, H. (1991). A novel method for in situ screening of yeast colonies with the beta-glucuronidase reporter gene. *Current Genetics*, *20*(5), 437-9.
29) Fatemi, M., Pao, M., Jeong, S., Gal-Yam, E., Egger, G., Weisenberger, D. et al. (2005). Footprinting of mammalian promoters: use of a CpG DNA methyltransferase revealing nucleosome positions at a single molecule level. *Nucleic Acids Research*, *33*(20), e176.